Chapter 4

Isolation of functional membrane vesicles of *Pyrococcus furiosus*

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Summary

Vesicles formed from cytoplasmic membranes are ideal systems to study the properties of solute transport systems without the influence of metabolic activities of the cell. A closed membrane vesicle system was obtained of the hyperthermophilic archaeon *Pyrococcus furiosus* by fusing its isolated membranes treated with chaotropic agents with liposomes made from tetra-ether lipids. These fused membrane vesicles have been used for the study of maltose transport.

Introduction

Information about the kinetics and specificity of solute transport systems are difficult to obtain from intact cells due to metabolism of the accumulated substrate by intracellular enzymes. Due to this metabolism solutes will not accumulate in whole cells to thermodynamic equilibrium and an analysis of the energetics of the transport process and even of the nature of the driving force is often difficult. Furthermore, the apparent kinetic parameters might be influenced by internally generated components and transport activities might be underestimated when the metabolic activity is high and the endproducts are secreted. As has first been shown by Kaback (Kaback, 1968), closed right-side-out and inside-out membrane vesicles represent ideal systems to study transport as such systems are devoid of the soluble metabolizing enzymes. Therefore, these systems are used widely to study solute transport and other membrane associated processes. Solute transport has been studied in hyperthermophilic archaea, but so far such studies have not been done in functional membrane vesicles.

Chapter 4

Pyrococcus furiosus, a hyperthermophilic, anaerobic archaeon, can grow heterotrophically on some sugars, such as starch, maltose and cellobiose (Fiala and Stetter, 1986). These sugars are rapidly metabolized. Information about carbohydrate transport in this organism has so far been obtained from studies in whole cells (Koning *et al.*, 2001; Koning *et al.*, 2002b).This manuscript describes the isolation of a functional membrane vesicle system of *Pyrococcus furiosus* that will allow more detailed studies on substrate transport.

Results

Preparation of membrane vesicles

For the preparation of *Pyrococcus furiosus* membranes, cells were broken using French Press and the membrane fraction was recovered by ultracentrifugation. Attempts were made to generate a valinomycin-induced potassium diffusion potential and to follow the resulting $\Delta\psi$ in time with the fluorescent probe $DiSC_3(5)$ to test the leakage of the *P. furiosus* membranes especially for protons. However a $\Delta\psi$ could not be recorded under these conditions (Fig. 1A). In an attempt to obtain closed vesicles, the *P. furiosus* membranes were fused with liposomes composed of tetraether lipids derived from *Sulfolobus acidocaldarius*. These liposomes have been found to be very impermeable to protons (van de Vossenberg *et al.*, 1995), even at high temperatures. However, also these fused membranes were unable to maintain an imposed $\Delta\psi$ (Fig. 1B), in contrast to the nonfused tetraether liposomes (Fig. 1C). Since archaeal cells are surrounded by an S-layer made from glycoproteins, it was anticipated that this sturdy protein layer

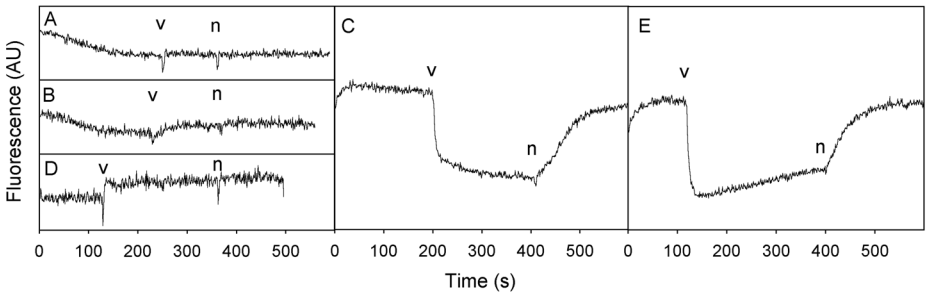

Figure 1. Proton permeability in *P. furiosus* membranes (A), *P. furiosus* membranes fused with liposomes (B), *S. acidocaldarius* tetraether liposomes (C), carbonate-treated *P. furiosus* membranes (D), and carbonate-treated *P. furiosus* membranes fused with liposomes (E). At the indicated times, valinomycine (v) and nigericine (n) were added to create and dissipate a potassium-diffusion potential $\Delta\psi$.

around the membrane interferes with membrane vesicle closure. To remove (part of) the S-layer, the *P. furiosus* membranes were incubated with high concentrations of various chaotropic agents (Na_2CO_3, KSCN or ureum). Also, the membranes obtained by this treatment were still not able to maintain a $\Delta\psi$ (Fig. 1D). However, when these treated membranes were fused with tetraether liposomes, a closed system was obtained (Fig. 1E). The various chaotropic agents yielded similar results, and the alkaline carbonate treatment was chosen to routinely remove the peripheral proteins from the membrane vesicles.

To firmly establish that carbonate treated *P. furiosus* membranes indeed fused with the tetraether liposomes, the membrane preparation was analysed on a sucrose gradient. The liposomes and *P. furiosus* membranes migrated differently in the gradient. Tetraether lipids have a brownish colour and migrate at a sucrose concentration of about 30 %, while *P. furiosus* membranes were recovered in the 47 % sucrose layer. The fused membranes yielded a single band that migrated at a sucrose concentration of 37 %, in between that of the *P. furiosus* membranes and tetraether liposomes. The identity of the various membrane fractions was confirmed by protein analysis, SDS-PAGE and measurements of the fluorescence after labeling the *P. furiosus* membranes and liposomes with the fluorophores R_{18} and N-NBD-PE, respectively (Fig. 2). Not all *P. furiosus* membranes were recovered in the fused membrane fraction. However, when a lower ratio of *P. furiosus* membranes to tetraether liposomes was used, the fraction of the *P. furiosus* membranes recovered in the fused membranes increased (Fig. 3).

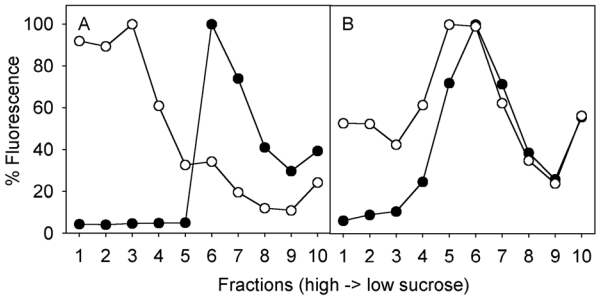

Figure 2. Sucrose gradient centrifugation of N-NBD-PE containing tetraether liposomes and carbonate-treated membranes before (A) and after (B) freeze/thawing induced fusion. ○, NBD fluorescence; ●, protein content.

The *S. acidocaldarius* lipids used for the fusion consist solely of tetraether lipids (Langworthy *et al.*, 1974), while *P. furiosus* membranes contain both di- and tetraether lipids (Fiala and Stetter, 1986). To study the influence of a different lipid composition, liposomes of *P. furiosus* lipids were also fused with *P. furiosus* membranes. Based on sucrose gradient centrifugation analysis, no difference could

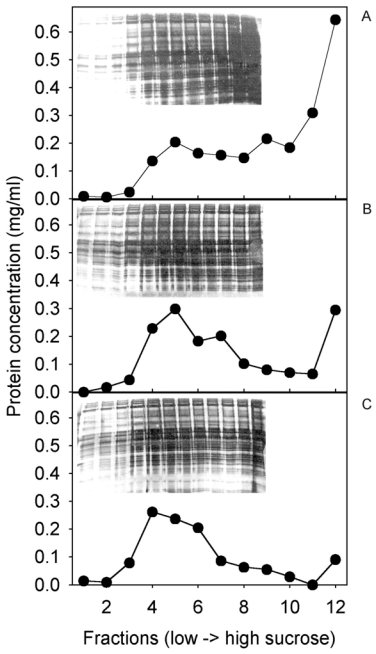

Figure 3. Influence of the *P. furiosus* membrane to liposome ratio on the fusion efficiency. (A) 1:1, (B) 1:5, and (C) 1:10 (mg of *P. furiosus* membrane protein-to-mg of tetraether lipid). Sucrose gradients were fractionated and the protein content was determined. Insert: silver-stained SDS-PAGE of the indicated sucrose gradient fractions.

be observed in the efficiency of fusion of the *P. furiosus* membranes with liposomes composed of *S. acidocaldarius* or *P. furiosus* lipids (results not shown). Although *P. furiosus* grows at higher salt concentrations than used in these assays, a concentration of NaCl up to 0.5 M had no effect on the fusion efficiency.

Transport by membrane vesicles

Transport studies were performed in *S. acidocaldarius* tetraether lipid liposomes and fused membranes derived from maltose-grown *P. furiosus* cells. Liposomes and fused membranes were loaded with [^{14}C]-lactose, a substrate that is not transported by *P. furiosus*, and efflux of the label was followed in time at 80 ^{o}C. Upon dilution, no release of [^{14}C]-lactose was observed from the liposomes (Fig. 4A), nor from the fused membranes (Fig. 4B), unless the liposomes or membranes were solubilized with Triton X-100. Also in the presence of ADP or ATP no release of lactose from the fused membranes was observed (Fig. 4B).

Membrane vesicles loaded with [^{14}C]-maltose did not show release of maltose upon the addition of ADP (Fig. 4C) or the nonhydrolysable ATP analog AMP-PNP (results not shown). In contrast, addition of ATP caused a small, but significant release of [^{14}C]-maltose (Fig. 4C). ATP-dependent release of maltose was not observed in tetraether liposomes loaded with [^{14}C]-maltose (Fig. 4A).

The observed transport activity did not differ significantly when the tetraether lipid fraction in the fused membranes was increased (data not shown). Strikingly, [^{14}C]-maltose was not released upon addition of Triton X-100 and even measured to the original loading level. This result is most likely explained by the presence of maltose binding protein present both at the inner and outer surface of the fused membranes. In the presence of ATP,

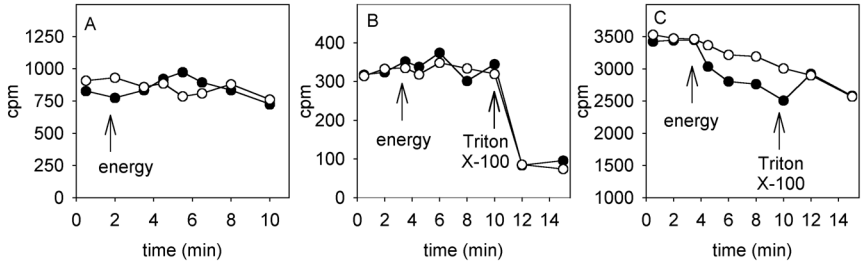

Figure 4. [^{14}C]-maltose (A and C) and [^{14}C]-lactose (B) release from liposomes (A) or fused membranes (B and C) after the addition of ADP (○) or ATP (●). Triton X-100 (0.05 %) was added to solubilize the fused membranes.

the binding proteins located at the inner surface of the membrane most likely unload maltose, but upon the addition of Triton X-100, the released maltose presumably rebinds to these binding proteins. The high concentration of membrane-associated maltose binding protein results in a high background binding. The non-fused *P. furiosus* membranes were removed from the fused membranes by sucrose gradient centrifugation. These purified membrane vesicles did not show an improved maltose efflux activity.

Maltose uptake by membrane vesicles

In a different assay, uptake of [^{14}C]-maltose was studied by fused membranes containing ATP in the inner lumen. After removal of external nucleotides, membranes were pre-heated at 80 ^{o}C, and [^{14}C]-maltose was added to start the transport process. Non-loaded and ADP-loaded fused membranes were used as controls. The increase of signal in ADP-loaded or non-loaded membrane vesicles is due to the presence of maltose binding protein on the outer surface of the vesicles. However, the ATP-loaded fused membranes showed a significant higher level of [^{14}C]-maltose uptake (Fig. 5A). As expected in this experiment, upon the addition of Triton X-100, the ATP-stimulated accumulated [^{14}C]-maltose was released. Addition of ATP to the outside of the fused membranes also resulted in a slight decrease of accumulated maltose, suggesting some maltose efflux (Fig. 5B).

Discussion

Membrane vesicles form an ideal system to study substrate transport processes. Here we report on the attempts to construct functional membrane vesicles from the hyperthermophilic archaeon *P. furiosus*. A closed membrane system could

be obtained only when carbonate-treated membranes were fused with liposomes composed of ether lipids derived from *S. acidocaldarius* or *P. furiosus*. With these fused membranes, a low but significant level of maltose transport could be observed when the system was supplemented with ATP. In contrast, liposomes or nonfused *P. furiosus* membranes did not support this transport activity. *P. furiosus* membranes presumably do not form sealed membrane vesicles and therefore are unable to retain accumulated maltose. Maltose transport activity was observed both by ATP-dependent release of [^{14}C]-maltose from the vesicle lumen as well as ATP-dependent [^{14}C]-maltose uptake by fused membranes. This suggests that the membrane fragments of *P. furiosus* are present in both right-side out and inside out orientation in the fused vesicles. This scrambled orientation is presumably caused by the fusion and sonication procedure. As a consequence, the levels of [^{14}C]-maltose transport by these fused membranes are too low. The presence of high affinity maltose binding protein at both faces of the membrane interferes with the transport assays. Both findings make these membrane vesicles less suited for detailed transport studies.

Experimental procedures

Organism and growth conditions

P. furiosus Vc1 (DSM 3638) (Fiala and Stetter, 1986) was grown at 80 ºC in modified *Methanococcus* medium (Kengen *et al.*, 1993) under anaerobic conditions in the presence of 5 mM of the indicated carbohydrate. *Sulfolobus acidocaldarius* DSM 639 was grown routinely in a 50 l fermentor at 80 ^{o}C under aerobic conditions (van de Vossenberg *et al.*, 1995).

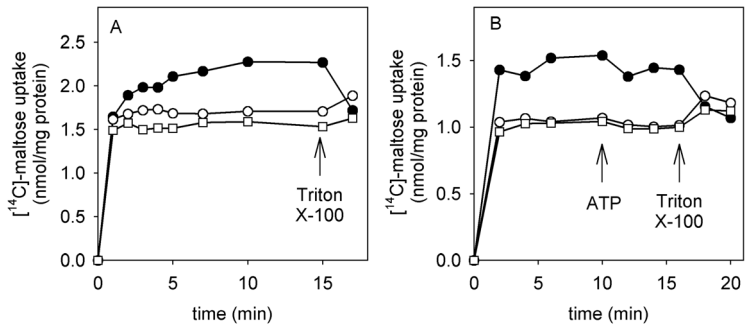

Figure 5. Uptake of [^{14}C]-maltose by fused membrane vesicles loaded with ATP (●), ADP (○), and non-loaded membrane vesicles (□) in the absence (A) or presence (B) of external ATP. Triton X-100 (0.05 %) was added to solubilize the fused membranes.

Membrane isolation

Cells were after harvesting resuspended in 50 mM Tris-HCl pH 7.5, and broken by a single pass through a French Pressure cell at 600 lb/in^2. Membranes were collected by centrifugation for 45 min at 100,000 x g at 4^{o}C. The pellet was resuspended in 50 mM Tris-HCl pH 7.5 and stored at –80 ^{o}C until use. When required, membranes were treated with chaotropic agents to remove peripheral membrane proteins. For this purpose, the membranes were resuspended in either 6 M ureum in 10 mM Tris pH 8.0, 44 mM Na_2CO_3 pH 10, or 1 M KSCN in 10 mM Tris pH 8.0 and 5% DMSO as indicated. After incubation (30 min on ice, 20 min at 45 ^{o}C, and 60 min at 20 ^{o}C, respectively), membranes were collected by centrifugation (15 min at 350,000 x g at 4 ^{o}C) and washed with buffer (50 mM potassium phosphate or HEPES-KOH, pH 7.0).

Preparation of liposomes

Lipids were isolated from freeze-dried *S. acidocaldarius* or *P. furiosus* cells as described previously (van de Vossenberg *et al.*, 1995), dried by vacuum rotary evaporation, and hydrated in buffer (50 mM potassium phosphate or HEPES-KOH, pH 7.0) to a final concentration of 20 mg/ml. Liposomes were prepared by five consecutive freezing and thawing steps and subsequent sonication (three cycles of 15 s on, 45 s off).

Preparation of membrane vesicles

Fused membrane vesicles were made by mixing an equal volume of liposomes (20 mg/ml) with carbonate-treated membranes (20 mg of protein/ml), two consecutive freezing (into liquid nitrogen) and thawing steps and subsequent sonication (three cycles of 15 s on, 45 s off). When indicated, a different ratio of lipids to membrane vesicles was used for the fusion step.

Measurements of the membrane potential

The integrity of membrane vesicles, liposomes and fused membrane vesicles was tested by the ability to maintain an imposed membrane protential, $\Delta\psi$. The $\Delta\psi$ was followed with the fluorescent probe diethylthiadicarbocyanine iodide ($DiSC_3(5)$), as described (Singh *et al.*, 1985). Fluorescence measurements were performed in a Perkin Elmer Luminescence Spectrometer LS 50B at 70 ^{o}C, using a thermostated, magnetically stirred sample compartment.

Transport assays

Fused membrane vesicles were loaded with 0.5 mM ADP or ATP (final concentration) in the presence of 1 mM $MgSO_4$ by freeze/thawing and sonication. To remove non-entrapped nucleotide, membrane vesicles were applied to a PD10 column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and eluted with HEPES-KOH, pH 7.0. The fused membrane vesicles (10 mg of protein/ml) were incubated in HEPES-KOH pH 7.0 at 80 ºC, and after 3 min, [^{14}C]-maltose (516 mCi/mmol; Amersham-Radiochemicals, Little Chalfont, Buckinghamshire, United Kingdom) was added to a final concentration of 5 µM. Samples of 100 µl were taken at various times, filtered over BA85 nitrocellulose (Protran; Schleicher & Schuell, Dassel, Germany), and washed twice with 2 ml 0.1 M LiCl. The radioactivity retained on the filters was determined by liquid scintillation counting.

For efflux studies, liposomes or fused membrane vesicles were loaded with [^{14}C]-maltose or [^{14}C]-lactose (516 mCi/mmol; Amersham, UK) to a final concentration of 5 µM by two consecutive freeze/thawing steps

and brief sonication (three cycles of 15 s on, 45 s off). The loaded liposomes or fused membrane vesicles were subsequently diluted 20-fold in preheated buffer (50 mM potassium phosphate, pH 7.0, 1 mM $MgSO_4$) of 80 ^oC. Samples (100 µl) were taken before and after the addition of ADP, ATP or AMP-PNP (0.5 mM final concentration). Triton X-100 was added to a final concentration of 0.05% to release the remaining label. Retention of the radiolabel was measured by filtration as described above.

Sucrose gradient centrifugation

Sucrose was dissolved in buffer (50 mM potassium phosphate, pH 7.0) to the appropriate concentrations. A step gradient of 1 ml of 25, 30, 35, 40, 45, and 50 % (w/v) sucrose was made, and the membrane vesicles, cell membranes or liposomes were applied on top of the gradient. Samples were centrifuged for 16 h at 15 ^oC at 200,000 x g in a swing-out rotor (Beckman TLS-55). Subsequently, the gradient was fractionated from the top (100 µl fractions) and analysed on SDS-PAGE and for protein content (Bradford, 1976). Fractions containing the membrane vesicles were washed in buffer and used immediately.

For lipid detection, membrane vesicles were labeled with the fluorophore R_{18} (Molecular Probes, Leiden, The Netherlands), while liposomes were labeled with N-NBD-PE that was incorporated during preparation (Molecular Probes, Leiden, The Netherlands). Membranes (5 mg of protein/ml) were mixed with 50 nmol R_{18} (in 5 µl ethanol) and incubated at room temperature for 1 h in the dark. N-NBD-PE (60 µl; 1 mg/ml) and lipids (1 ml; 10 mg/ml) were mixed in chloroform-methanol (3:1) and vacuum-dried as a lipid film. The lipid film was hydrated and converted into liposomes as described above. After sucrose centrifugation of the membranes or liposomes, the fluorescence of R_{18} and N-NBD-PE were measured at excitation wavelengths of 560 or 475 nm, and emission wavelengths of 590 or 530 nm, respectively.